Available online at www.sciencedirect.com

SCIENCE DIRECT

Virology 324 (2004) 74–83

VIROLOGY

www.elsevier.com/locate/yviro

Pseudotype hepatitis C virus enters immature myeloid dendritic cells through the interaction with lectin

Aki Kaimori,[a] Tatsuya Kanto,[a,b] Chang Kwang Limn,[c] Yasumasa Komoda,[c] Chika Oki,[a] Michiyo Inoue,[a,b] Hideki Miyatake,[a] Ichiyo Itose,[a] Mitsuru Sakakibara,[a] Takayuki Yakushijin,[a] Tetsuo Takehara,[a] Yoshiharu Matsuura,[c] and Norio Hayashi[a,*]

[a] *Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan*
[b] *Department of Dendritic Cell Biology and Clinical Application, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan*
[c] *Research Center for Emerging Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan*

Received 14 October 2003; returned to author for revision 12 November 2003; accepted 24 March 2004

Abstract

Dendritic cells (DC) are the most potent antigen-presenting cells that regulate immune responses. One of the mechanisms for hepatitis C virus (HCV) persistence is the ability of HCV to suppress DC function. Direct HCV infection to blood DC has been implicated for DC dysfunction. To clarify the susceptibility of each DC subset to HCV, we used pseudotype vesicular stomatitis virus (VSV) coated with chimeric HCV envelope glycoproteins (E1 and E2). We demonstrate that pseudotype VSV enters myeloid DC (MDC) but not plasmacytoid DC (PDC). The highest efficiency of pseudotype VSV entry to MDC was observed when MDC were cultured with GM-CSF. Such efficiency decreased when MDC are matured with the treatment of IL-4, CpG oligodeoxynucleotide, or CD40 ligand. Mannan inhibited pseudotype VSV entry to MDC, but Ca^{2+} chelators failed to do so. These results show that pseudotype VSV possessing HCV-E1 and E2 enters immature MDC through the interaction with lectins in a Ca^{2+}-independent manner.

Keywords: Hepatitis C virus; Envelope proteins; Pseudotype virus; Dendritic cells; Lectin

Introduction

Hepatitis C virus (HCV), a single-stranded plus-sense RNA virus belonging to the fraviviridae family (Miller and Purcell, 1990), causes persistent infection in more than 70% of infected patients. The most important feature of HCV persistence is the potential for liver disease progression from mild hepatitis to liver cirrhosis and hepatocellular carcinoma (HCC) (Alter et al., 1989). Chronic HCV infection is a serious health problem because the total number of HCV-positive HCC patients is growing worldwide. One of the mechanisms for HCV persistence is the ability of HCV to escape from the host cellular immune response (Farci et al., 1992; Weiner et al., 1995). Cumulative studies show that functional impairment of immunocompetent cells is found in patients with chronic HCV infection (Corado et al., 1997; Wedemeyer et al., 2002), suggesting that HCV has various arms for suppressing the immune response.

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) that regulate various immune responses (Banchereau and Steinman, 1998; Hart, 1997). Blood DC mainly consist of two subsets, that is, myeloid and plasmacytoid DC (Liu, 2001). Myeloid DC (MDC) are characterized by their potent immunostimulatory properties for both primary and secondary T-cell responses against virus. Plasmacytoid DC (PDC), previously known as interferon (IFN)-producing cells, produce a large amount of type I IFN upon virus infection (Liu, 2001). However, some viruses such as

* Corresponding author. Department of Molecular Therapeutics, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita, Osaka 565-0871, Japan. Fax: +81-6-6879-3449.
E-mail address: hayashin@moltx.med.osaka-u.ac.jp (N. Hayashi).

0042-6822/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2004.03.038

measles virus or HIV have been shown to infect monocyte-derived DC (MoDC) or PDC and subsequently inhibit their immunostimulatory functions (Grosjean et al., 1997; Patterson et al., 2001; Schnorr et al., 1997). Previous studies including our own have shown that MoDC from patients with chronic HCV infection are functionally impaired (Bain et al., 2001; Kanto et al., 1999). In addition, our recent investigation revealed that the function of both types of blood DC is suppressed as well in HCV-infected patients (Kanto T. et al., unpublished data). These results led us to hypothesize that HCV infection to DC is one of the mechanism for DC dysfunction in chronic hepatitis C patients.

The existence of the HCV genome in blood cells including DC has been shown in several studies by means of reverse transcription (RT)-PCR (Bain et al., 2001; Lerat et al., 1996, 1998; Navas et al., 2002). The detection of the positive strand of HCV-RNA does not enable to define whether HCV enters cells or only adheres to their surface. Alternatively, the negative strand of HCV-RNA has been used as a surrogate marker of HCV replication (Navas et al., 2002). Recently, Matsuura et al. (2001) established the pseudotype vesicular stomatitis virus (VSV) having chimeric HCV E1 and E2 protein as an envelope (VSV-E1E2). Because it has a green fluorescent protein (GFP) reporter gene in its genome, the infected cells can be viewed under fluorescence. Using this system, we tried to clarify the susceptibility of each DC to HCV. Consequently, we demonstrate that MDC is susceptible to VSV-E1E2 but PDC is not. Furthermore, we showed that the lectin-containing molecules on MDC are critically involved in VSV-E1E2 entry. Our study provides useful information for the exploration of target molecules that efficiently block HCV entry to DC.

Results

Immature MDC are susceptible to VSV-E1E2

We inoculated pseudotype VSV on various cells separated from PBMC or cord blood. Because no positive fluorescence was obtained from CD4 T cells, CD8 T cells, B cells, NK cells, and fresh PDC inoculated with VSV ΔG-G which is complemented with the VSV G protein, the susceptibility of these cells to VSV-E1E2 could not be estimated. In contrast, VSV ΔG-G entered fresh MDC, monocytes, and $CD34^+$ hematopoietic precursor cells on the day of separation, whereas VSV-E1E2 and VSV ΔG did not. Thus, fresh MDC as well as DC precursors are not susceptible to VSV-E1E2 (Fig. 1).

To examine the influence of differentiation or maturation of DC on the susceptibility to the pseudotype VSV, MDC were cultured in the presence of GM-CSF with or without IL-4. Phenotypic analysis revealed that day 4 MDC cultured with GM-CSF and IL-4 had higher expression of CD1a and CD86 than those cultured with GM-CSF only (Fig. 2), showing the role of IL-4 in DC maturation. No significant difference was observed in the expression of CD11c, CD40, CD80, CD83, and HLA-DR between these MDC (Fig. 2). On day 4 of culture in the presence of IL-3, PDC showed higher expressions of CD40, CD80, CD83, and CD86 when compared to the day of separation (data not shown).

After the inoculation with VSV ΔG-G, positive signals were obtained from day 4 MDC and day 4 PDC, regardless of the difference of cytokines used (Figs. 3A and B). No significant signals were detected from day 4 MDC and day 4 PDC inoculated with VSV ΔG. With respect to VSV-E1E2, GFP^+ cells were observed in day 4 MDC but not in day 4 PDC (Figs. 3A and B). In

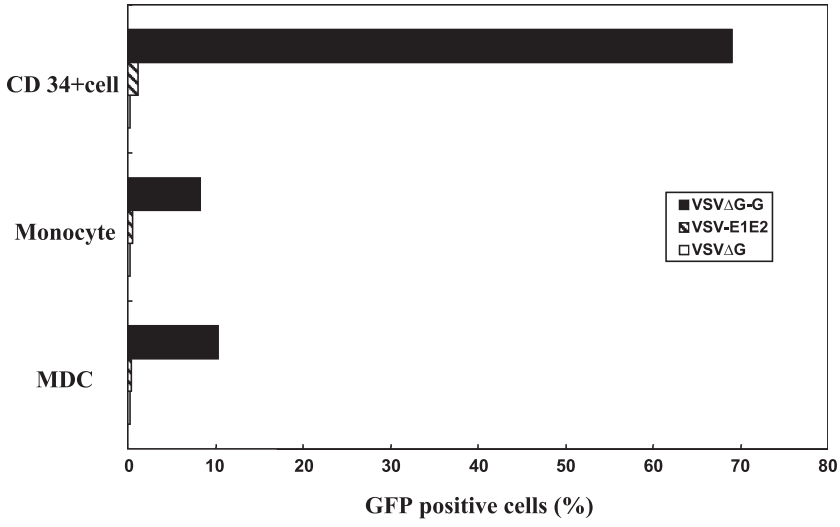

Fig. 1. Freshly isolated DC are not susceptible to VSV-E1E2. Freshly isolated $CD34^+$ hematopoietic precursor cells, monocytes, or MDC were inoculated with pseudotype VSVs and the percentages of GFP^+ cells were determined by flow cytometry. Representative results from three experiments are shown.

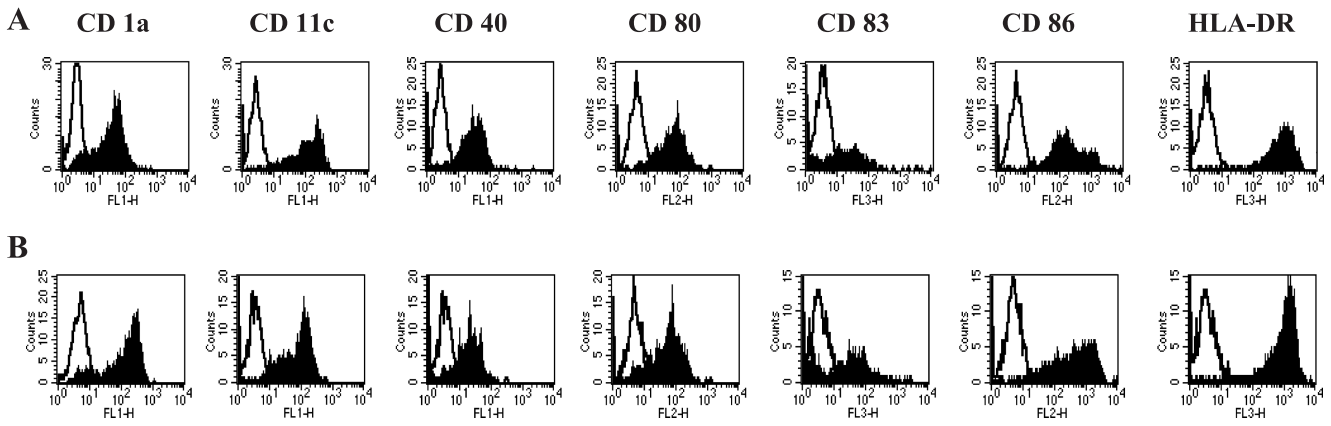

Fig. 2. Phenotypes of MDC cultured with GM-CSF or GM-CSF and IL-4. Flow cytometric analyses of surface molecules on day 4 MDC, obtained from healthy volunteers, cultured with GM-CSF (A) or with GM-CSF and IL-4 (B). Representative results from three subjects are shown. Open histograms represent the results with isotype Ab and filled ones represent those with relevant Abs. Fluorescence intensity is shown in the *x*-axis and the number of cells is shown in the *y*-axis.

comparison of the culture conditions for MDC, higher percentage of GFP^+ cells was observed in day 4 MDC cultured with only GM-CSF than those with GM-CSF and IL-4 (Fig. 3B).

To confirm the reliability of the pseudotype VSV system in the assessment of E1E2-mediated virus entry, we quantified HCV-RNA in day 4 cultured MDC or PDC after inoculation of a window-period serum from a hepatitis C patient. Among the cells examined, the highest HCV RNA titer was detected in day 4 MDC cultured with GM-CSF (Fig. 3C), which was compatible with the results obtained with the pseudotype VSV. In contrast with the results of VSV-E1E2 inoculation, low-level HCV RNA were detected by quantitative RT-PCR in PDC inoculated with authentic HCV (Fig. 3C). To further investigate whether HCV replicates in each DC subset after HCV inoculation, we performed strand-specific RT-PCR for the detection of negative-strand HCV-RNA as a surrogate marker of HCV replication. Positive strand of HCV-RNA was detected both in MDC cultured with GM-CSF and PDC with IL-3, whereas negative strand was detected in GM-CSF-MDC but not in IL-3-PDC (Fig. 3D). These results suggest that HCV replicates in GM-CSF-MDC but not in IL-3-PDC. Therefore, the data with the pseudotype VSV system correctly reflect the susceptibility of cells to authentic HCV.

Maturation stimuli protect MDC from VSV-E1E2

Based on the findings described above, we hypothesized that the more MDC mature, the less susceptible they are to VSV-E1E2. To find the substances protecting DC from HCV infection, we treated MDC with various maturation factors for the inoculation study. In MDC cultured with GM-CSF, the addition of IL-4, CpG oligodeoxynucleotide (ODN) 2006, or CD40 ligand (CD 40L) to the culture significantly reduced the percentage of GFP^+ cells with VSV-E1E2 without influencing their susceptibility to VSV ΔG-G (Fig. 4). On the other hand, IFN-α, polyI:C, TNF-α, and lipopolysaccharide (LPS) reduced the percentage of GFP^+ cells with both VSV-E1E2 and VSV ΔG-G (Fig. 4). Phenotypic analysis revealed that IL-4 up-regulated the expression of CD1a and CD86 on MDC cultured with GM-CSF (Fig. 2). CpG ODN or CD40L also up-regulated the expression of CD1a, CD83, and CD86 on MDC cultured with GM-CSF (data not shown). Therefore, immature DC lose their susceptibility to VSV-E1E2 as they develop to be more mature state.

Lectin on DC is involved in VSV-E1E2 entry to DC

The C-type lectins expressed on DC are reported to interact with various viruses as well as microbial agents (Geijtenbeek et al., 2000; Tailleux et al., 2003). These studies led us to consider the involvement of lectins in VSV-E1E2 entry to DC. Thus, we first used mannan to examine whether it inhibits VSV-E1E2 entry to MDC. The pretreatment of MDC with mannan reduced the percentage of GFP^+ cells with VSV-E1E2 in a dose-dependent manner without having any impact on VSV ΔG-G entry (Fig. 5A). Such an inhibitory effect of mannan was confirmed with MDC inoculated with authentic HCV (data not shown). A D-mannose-specific lectin, methyl α-D-mannopyranoside (Kaku et al., 1991), also inhibited VSV-E1E2 entry to MDC in a dose-dependent fashion at concentrations from 10 to 40 μg/ml (data not shown). In contrast, galactose had no effect on the infection with either VSV-E1E2 or VSV ΔG-G in MDC (Fig. 5A). Interestingly, EDTA did not reduce the infectivity of VSV-E1E2, whereas it completely abolished that of VSV ΔG-G (Fig. 5A). These data demonstrate that mannose-type carbohydrate is involved in the interaction of DC with VSV-E1E2 in a Ca^{2+}-independent manner. The treatment of MDC with antihuman DC-SIGN Ab,

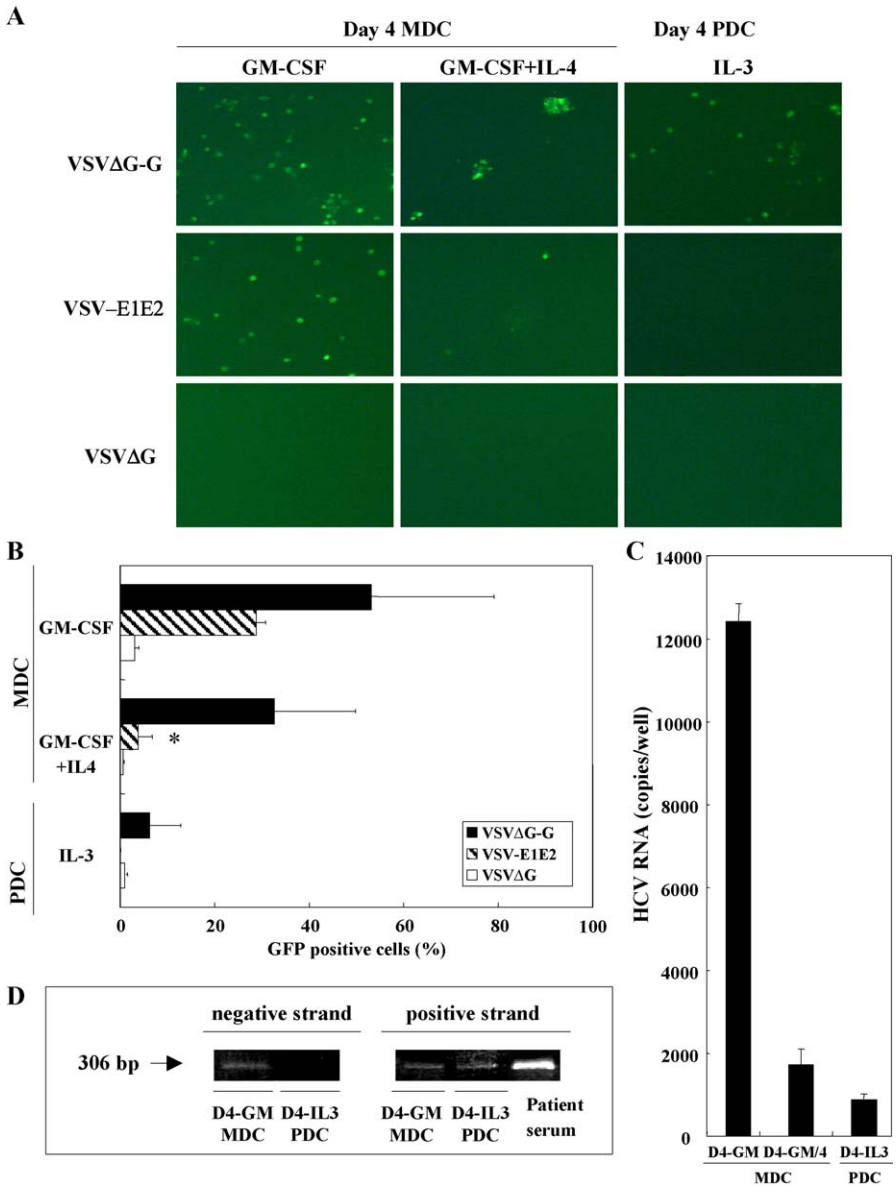

Fig. 3. Myeloid DC cultured with GM-CSF are susceptible to VSV-E1E2 or authentic HCV from patient serum. Day 4 MDC cultured with GM-CSF or with GM-CSF and IL-4 or day 4 PDC cultured with IL-3 were inoculated with VSV ΔG, VSV ΔG-G, or VSV-E1E2. They were viewed under fluorescence microscopy (A) and the percentages of GFP^+ cells were analyzed by flow cytometric analysis (B). The results of fluorescence microscopy are the representative ones from three subjects. The results of flow cytometric analysis are expressed as the mean + SD from three representative experiments. $*P < 0.001$ vs. VSV-E1E2-inoculated day 4 MDC cultured with GM-CSF. (C) Quantitative analysis of HCV RNA in DC inoculated with HCV-positive patient serum was performed as described in Materials and method. D4-GM or D4-GM/4 represents MDC cultured with GM-CSF or GM-CSF and IL-4 for 4 days. D4-IL 3 represents PDC cultured with IL-3 for 4 days. The results are expressed as the mean + SD of triplicate wells from three representative experiments. (D) The detection of positive and negative strand of HCV-RNA in DC inoculated with HCV-positive patient serum. Strand-specific RT-PCR was performed with samples from MDC, PDC, and patient serum used as inoculum, as described in Materials and method. D4-GM and D4-IL 3 represent as the same as above.

which is able to block the binding of DC-SIGN to ICAM-3 (Wu et al., 2002), did not inhibit the entry of either VSV-E1E2 or VSV ΔG-G (Fig. 5B). To see whether the expression of DC-SIGN on MDC parallels their susceptibility to VSV-E1E2, we compared the expression of DC-SIGN between MDC cultured with GM-CSF and those with a combination of GM-CSF and IL-4. The expression of DC-SIGN was higher on MDC cultured with GM-CSF and IL-4 than on those with GM-CSF (Fig. 5C), which is contrary to their susceptibility to VSV-E1E2. These results show that DC-SIGN is less likely to be involved in the VSV-E1E2 entry to MDC. Human hepatoblastoma cell line, HepG2, is one of the most sensitive cells to pseudotype VSV (Matsuura et al., 2001). To compare the machinery of VSV-E1E2 entry between MDC and HepG2, we inoculated it to mannan-treated HepG2. In contrast to MDC, mannan did not inhibit the VSV-E1E2 entry to HepG2 (Fig. 5D), suggest-

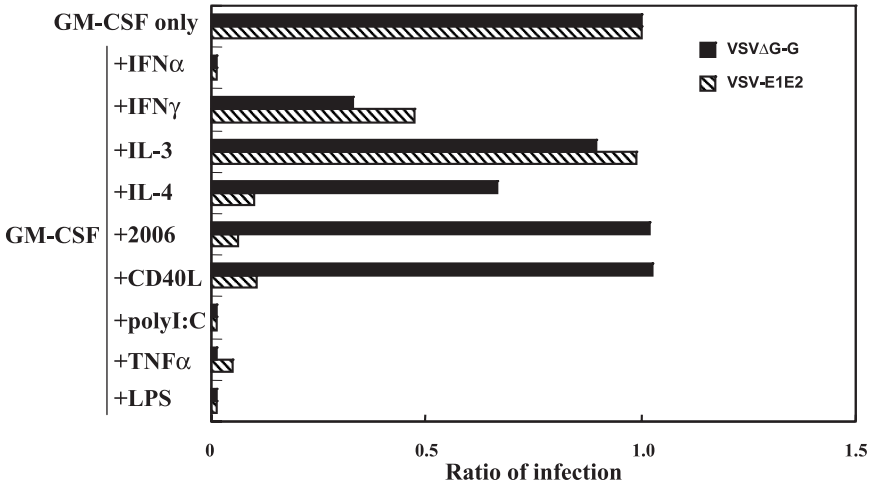

Fig. 4. IL-4, CpG ODN, or CD40L protects MDC from VSV-E1E2. Various immunomodulators were added to MDC cultured with GM-CSF, and the ratio of infection was determined between the cells treated with or without the reagents. IFN-α (100 U/ml), IFN-γ (100 U/ml), IL-3 (50 ng/ml), or IL-4 (10 ng/ml) was added on the day of MDC separation. CpG ODN 2006 (10 μM), CD40L (1 μg/ml), polyI:C (50 μg/ml), TNFα (20 ng/ml), or LPS (10 μg/ml) was added to MDC 24 hrs before the pseudovirus inoculation. Representative results are shown from three independent experiments.

ing that the molecules responsible for VSV-E1E2 entry differ between HepG2 and MDC. Furthermore, it also shows that mannan affects the molecules on MDC but not those on VSV-E1E2.

Discussion

Using the pseudotype VSV system, we have demonstrated that each DC subset has distinct susceptibility to HCV. First, VSV-E1E2 enters MDC but not PDC, which is in sharp contrast with PDC susceptibility to HIV (Patterson et al., 2001). Second, MDC cultured with GM-CSF are more susceptible to VSV-E1E2 than freshly prepared MDC or those cultured with GM-CSF and IL-4, showing that HCV targets immature MDC. Third, certain molecules containing the lectin domain on MDC are involved in the interaction with VSV-E1E2.

One of the suggested mechanisms of persistent HCV infection is the functional suppression of immunocompetent cells, including NK cells, T cells, and DC (Bain et al., 2001; Corado et al., 1997; Kanto et al., 1999; Wedemeyer et al., 2002). The possibility being raised for such immunological impairment is that HCV directly infects these blood cells. To elucidate this issue, investigators have used RT-PCR to examine whether the HCV genome is detectable or not in blood cells recovered from HCV-infected patients (Bain et al., 2001; Lerat et al., 1996, 1998). However, the existence of HCV-RNA does not enable to define whether HCV enters cells or only adheres to their surface. Instead of qualitative RT-PCR, we used the pseudotype VSV system to study the HCV E1E2-mediated virus entry to each DC subset. The pseudotype VSV system is a valid model for investigating the early steps of HCV infection, that is, viral attachment, receptor binding, and membrane fusion. Also, it enables us to estimate the efficiency of HCV E1E2-mediated virus entry to target cells. However, there are several limitations in this system. First, the positive results with pseudotype VSV do not indicate the replicative ability of HCV in the relevant cells. Because pseudotype VSV is constructed from VSV genome, their replication capacity is not exactly the same as HCV. Second, the evaluation of pseudotype VSV entry is possible only in cells that permit VSV replication. In other words, it cannot be used to determine the entry of VSV-E1E2 in the cells that suppress VSV replication. In this study, we could not evaluate the susceptibility of T, B, NK cells or fresh PDC to VSV-E1E2.

Alternatively, we performed an inoculation experiment with authentic HCV particle to confirm the reliability of the pseudotype VSV system. Quantitative RT-PCR assay showed that the highest titer of HCV-RNA was detected in MDC cultured with GM-CSF; however, low titer of HCV-RNA was detected in PDC. We hypothesized that the reason such discrepancy occurs between two assays is that RT-PCR amplified HCV genome from HCV attached to the surface of PDC. Strand-specific RT-PCR showed that negative strand of HCV-RNA, a surrogate marker of HCV replication, was detected in MDC cultured with GM-CSF but not in PDC. These results indicate that HCV enters and replicates in MDC but not in PDC, which are well correlated with those of pseudotype VSV entry.

The Th1 response is thought to be needed to eradicate HCV from hosts (Gerlach et al., 1999). Myeloid DC potentially activate CD4+T cells to support Th1 differentiation (Liu, 2001). We found that MDC from HCV-infected patients are less able to induce the Th1 response than the normal counterpart (Kanto T., unpublished data). It has been reported that MoDC expressing HCV protein were impaired in the stimulation of allogeneic T cells and IL-12 production, indicating an inhibitory capacity of HCV

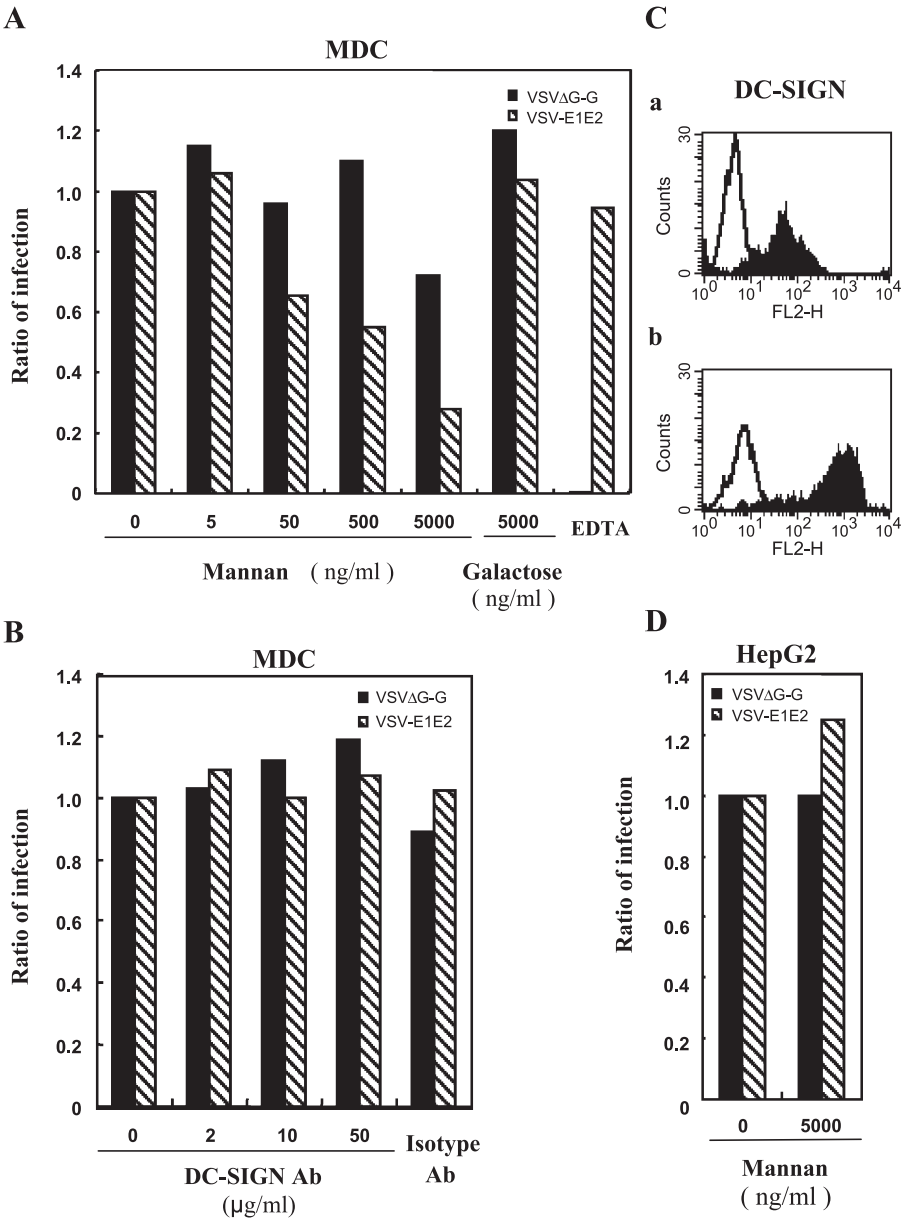

Fig. 5. Mannan inhibits VSV-E1E2 entry to MDC, but not to HepG2. Various concentrations of mannan, galactose, or 5 mM EDTA were added to day 4 MDC cultured with GM-CSF before the pseudotype VSV inoculation. Alternatively, day 4 MDC were treated with 2, 10, or 50 μg/ml antihuman DC-SIGN Ab or 10 μg/ml isotype IgG_{2B} for 30 min before the pseudotype VSV inoculation. HepG2 was treated with 5 μg/ml mannan before the addition of pseudotype VSV. The ratio of infection with pseudotype VSV in MDC (A, B) or HepG2 (D) was determined as described in Materials and method. Representative results are shown from three independent experiments. (C) Flow cytometric analyses were done for DC-SIGN expression on day 4 MDC cultured with GM-CSF (a) or with GM-CSF and IL-4 (b) generated from a healthy volunteer. Representative results from three subjects are shown. Open histograms represent the results with isotype Ab and filled ones represent those with anti-DC-SIGN Abs. Fluorescence intensity is shown in the *x*-axis and the number of cells is shown in the *y*-axis.

protein on DC function (Sarobe et al., 2002). These data suggest that direct HCV infection to myeloid DC suppress their function. Thus, the protection of DC from HCV infection is a rational approach to improve DC-mediated anti-HCV immune response. In the present study, we demonstrate that some of the maturation stimuli are capable of protecting DC from VSV-E1E2 entry. However, MoDC from HCV-infected patients are reported to be resistant to maturation stimuli, such as TNF-α (Auffermann-Gretzinger et al., 2001). Thus, further investigation is necessary to determine the effective modulation that allows MDC to mature in HCV infection. As shown in this study, CpG ODN or CD40L stimulated MDC to mature and become less susceptible to VSV-E1E2. The potent ability of CpG ODN to stimulate a DC-inducing Th1 response has been demonstrated in vivo tumor treatment models (Heckelsmiller et al., 2002). Therefore, CpG ODN are promising as a DC adjuvant in HCV-infected patients that potentially leads to MDC maturation as well as boosting Th1 response.

It is arguably necessary to identify molecules that are responsible for HCV entry to protect DC. Previously, tetraspanin CD81 has drawn much attention as a presumed HCV receptor due to its high affinity to HCV-E2 (Pileri et al., 1998). However, its involvement in VSV-E1E2 entry is unlikely because the CD81 is equally expressed on both MDC and PDC but is lacking on the VSV-E1E2-sensitive cell line HepG2 (Flint et al., 1999). In this study, we showed some of the characteristics of the molecules on DC involved in VSV-E1E2 entry. They are myeloid-lineage specific, inducible by GM-CSF, down-regulatable by IL-4 or other maturation stimuli. In addition, they possess some lectin domain, as evidenced by the inhibition of VSV-E1E2 as well as authentic HCV entry with mannan. These results raised the possibility that such molecules are categorized as members of C-type lectins, such as DC-SIGN, mannose receptor (MR), Langerin, DEC205, BDCA2, or asialoglycoprotein receptor. (Figdor et al., 2002) Recently, two independent studies have demonstrated that HCV E1 and E2 glycoproteins efficiently bind to DC-SIGN (Lozach et al., 2003; Pohlmann et al., 2003). However, the involvement of DC-SIGN in VSV-E1E2 entry is less likely because its expression on MDC did not parallel the susceptibility to VSV-E1E2. In addition, the treatment with anti-DC-SIGN Ab did not inhibit VSV-E1E2 entry. Furthermore, the treatment with EDTA failed to block VSV-E1E2 entry to MDC, showing that the VSV-E1E2 entry occurs in a Ca^{2+}-independent manner. It is still obscure in which step lectins are involved in VSV-E1E2 entry to MDC. From an analogy with the interaction of HIV with DC-SIGN (Geijtenbeek et al., 2000), it is conceivable that lectins are essential for HCV attachment to MDC. Nevertheless, the possibility remains that HCV entry receptors or co-receptors, which may be other than lectins, exist on MDC. With the aid of the pseudotype VSV system, exploration has been underway to identify the molecules on MDC that are critically involved in HCV infection.

Materials and method

Reagents

Recombinant human IL-4 and GM-CSF were purchased from PeproTech (London, UK). Recombinant human soluble CD40L, human TNF-α and IL-3, and mouse monoclonal antihuman DC-SIGN (CD209/DC-SIGN1) Ab (12507) were from R & D Systems (Minneapolis, MN). LPS, polyI:C, mannan, galactose, and methyl α-D-mannnopyranoside were from Sigma (St. Louis, MO). Recombinant human IFN-γ was from Strathman Biotech GmbH (Hamburg, Germany). Human lymphoblastoid IFN-α was provided by Sumitomo Pharmaceuticals (Osaka, Japan). Unmethylated CpG ODN 2006 (Krug et al., 2001) was synthesized at and purchased from Sigma Genosys (Hokkaido, Japan). Isotype IgG (mouse IgG_{2B}) for the blocking experiments was kindly provided from the JT laboratory (Osaka, Japan).

Separation of DC precursors and other cells from PBMC

After informed consent had been obtained from healthy volunteers, buffy coats were isolated from venous blood drawn from them at the Osaka Red Cross Blood Center (Osaka, Japan). PBMC were collected from buffy coats by Ficoll–Hypaque density-gradient centrifugation. B cells, MDC, and PDC were magnetically isolated by using CD19 microbeads, BDCA-1, or BDCA-4 DC isolation kits from Miltenyi Biotec (Bergish-Gladbach, Germany), respectively. BDCA-1^+ and BDCA-4^+ cells are phenotypically compatible with MDC and PDC, respectively (Dzionek et al., 2000). CD4, CD8 T cells, and NK cells were separated from PBMC by using the relevant Stem-Sep kits (Stem Cell Technologies Inc, Vancouver, BC). $CD34^+$ hematopoietic precursor cells were isolated from cord blood mononuclear cells by using CD34-microbeads from Miltenyi. The purity of all isolated cells was more than 90% as determined by FACS Caliber (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Culture of DC

Isolated MDC were cultured for 4 days in IMDM (GIBCO Laboratories, Grand Island, NY) supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, 10 mM Hepes buffer, and 10 μM nonessential amino acid (Complete medium, CM) containing 50 ng/ml GM-CSF with or without 10 ng/ml IL-4. PDC were cultured for 4 days in CM in the presence of 50 ng/ml of IL-3.

Flow cytometry

The expression of surface molecules on DC was analyzed by FACS Caliber (Becton Dickinson). For the staining, DC were stored with specific Abs or isotype Abs for 30 min at 4 °C in PBS containing 2% of BSA and 0.1% of sodium azide. The following FITC-, PE-, PerCP-, or PC5-conjugated antihuman mAbs were used: CD1a (NA1/34; DAKO, Glostrup, Denmark), CD11c (KB90; DAKO), CD14 (M5E2; Becton Dickinson), CD40 (5C3; BD Pharmingen, San Diego, CA), CD80 (L307.4; BD Pharmingen), CD83 (HB15a; Immunotech, Marseille, France), CD86 (IT2.2; B70/B7-2, BD Pharmingen), CDw123 (7G3; IL-3 receptorα chain, BD Pharmingen), DC-SIGN (120507; R & D Systems), and HLA-DR (L243; Becton Dickinson).

Assessment of pseudotype VSV entry into cells

To find which blood cells are susceptible to HCV infection, we used pseudotype VSV possessing chimeric HCV E1 and E2 protein which was generated as described

previously (Matsuura et al., 2001). The pseudotype VSV consists of recombinant VSV in which glycoprotein (G) gene is replaced with a reporter gene encoding GFP.

As an envelope, it possesses chimeric HCV E1 and E2 proteins (VSV-E1E2). The viruses were purified by centrifugation at 25 000 rpm for 2 h at 4 °C in SW28 rotor (Beckman Coulter Inc., Fullerton, CA) through 20% (v/w) and 60% (v/w) discontinuous sucrose gradient and were stored at −80 °C. To determine RNA copy numbers in the viral samples, TaqMan EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA) was used. We used forward and reverse primers (5′-cattattatcattaaaaggctc-3′ and 5′-gataaggtcaaatattccg-3′) that amplify a 323-bp segment of the pseudotyped VSV RNA and also used a dual fluorophore-labeled probe 5′-(6-carboxy-fluorescein)-atccagtggaatacccggcagattac-(6-carboxy-tetramethyl-rhodamine)-3′. The sequence detector (ABI Prism 7000, PE Applied Biosystems) allows measurement of the amplified products in indirect proportion to the increase in fluorescence emission continuously during the PCR amplification. The copy numbers in samples were determined based on the standard curve drawn by a known amount of in vitro synthesized pseudotyped VSV RNA. Because VSV efficiently replicates in a wide range of mammalian cells, we are able to determine the cells exhibiting susceptibility to pseudotype VSV by the expression of GFP. We used VSV ΔG which has no envelope protein as a negative control. Similarly, VSV ΔG-G was used as a positive control which is complemented with the VSV G protein. Various separated blood cells were prepared in CM at 5×10^4 cells/well on 96-well culture plates. Next, they were inoculated with the pseudotype viruses, VSV-E1E2 (1×10^{12} RNA copies/well), VSV ΔG (1×10^{12} RNA copies/well), or VSV ΔG-G (1×10^{11} RNA copies/well) and incubated for 16 h at 37 °C. The infected cells (GFP^+ cells) were observed under fluorescence microscopy, and their positive percentages were determined by FACS analysis. The net percentage of infected cells was expressed as % infection = (% of GFP^+ cells with VSV-E1E2 or VSV ΔG-G) − (% of GFP^+ cells with VSV ΔG).

To find the substances which potentially protect DC from HCV infection, we examined IFNα, IFNγ, IL-3, IL-4, CpG ODN 2006, CD40L, polyI:C, TNFα, or LPS for this purpose. The appropriate concentrations of these reagents were determined in a separate series of experiments. IFNα, IFNγ, IL-3, or IL-4 was added to DC on the day of separation. CpG ODN 2006, CD40L, polyI:C, TNFα, or LPS was added to DC 24 h before the inoculation of pseudovirus. To compare the inhibitory effect of reagents in VSV-E1E2 entry into cells, we determined the ratio of infection of cells with and without treatment.

DC express various molecules containing the lectin domain, some of which are reported to be essential for the attachment to virus (Figdor et al., 2002). To examine whether lectin-containing molecules on DC are involved in HCV infection, we tested mannan, methyl α-D-mannopyranoside, and galactose for the inhibition of VSV-E1E2 entry. Day 4 MDC cultured with GM-CSF were preincubated with various concentrations of mannan, methyl α-D-mannopyranoside, or galactose at 37 °C for 180 min and inoculated with the pseudotype VSV. We also treated DC with EDTA (5 mM), monoclonal antihuman DC-SIGN Ab (50, 10, or 2 μg/ml), or isotype IgG_{2B} before the pseudotype VSV inoculation. To compare DC with hepatoblastoma cell line, HepG2, we treated HepG2 with mannan before the inoculation.

Quantitative analysis of HCV RNA in cells inoculated with HCV particles from patient serum

To test the susceptibility of each DC subset to authentic HCV, we quantified HCV RNA in cells that had been inoculated with patient serum by means of real-time PCR. We used the commercial HCV seroconversion panel as an inoculum, which contains high HCV RNA titer (1×10^5 copies/μl) and no anti-HCV antibody (BioClinical Partners, Inc, USA). We added 3 μl/well of inoculum to DC on 96-well plates and incubated them at 37 °C for 24 h. DC were harvested and washed three times with IMDM supplemented with 1% FCS and then total RNA was extracted from DC using RNeasy Mini Kit (QIAGEN, Germany). To measure HCV RNA, TaqMan EZ RT-PCR kit (PE Applied Biosystems) was used. We used forward and reverse primers [5′-cgggagagccatagtgg-3′ (positions 130–146) and 5′-agtaccacaaggcctttcg-3′ (positions to 272 to 290)] that amplify a 161-bp segment of the 5′ noncoding region of HCV RNA and also used a dual fluorophore-labeled probe [5′-(6-carboxy-fluorescein)-ctgcggaaccggtgagtacac (positions 148–168)- (6-carboxy-tetramethyl-rhodamine) -3′]. The sequence detector (ABI Prism 7000) allows measurement of the amplified products in indirect proportion to the increase in fluorescence emission continuously during the PCR amplification. The copy number in the samples was determined based on the standard curve drawn by a known amount of in vitro synthesized HCV RNA.

The strand-specific RT-PCR assay for HCV-RNA in cells inoculated with authentic HCV particles

To detect negative-strand HCV-RNA that is indicative of RNA replication, we performed the strand-specific RT-PCR assay referring to the methods described by Navas et al. (2002) with some modifications. We used the same batch of HCV seroconversion panel as an inoculum as described in the above section. We added 3 μl/well of inoculum to DC on 96-well plates and incubated them at 37 °C for 24 h. After the washing of DC for three times, total RNA was extracted from DC as the same way as we did in quantitative RT-PCR. As a control for the detection of HCV-RNA, 9 μl of inoculum was used. We used sense and anti-sense primers [5′-cactcccctgtgaggaactactgtc-3′ (positions 38–62) and 5′-atggtgcacggtctacgagacctcc-3′ (positions 319–343) that amplify a 306-bp segment of the 5′ noncoding region of HCV

genome. Ten microliters of purified RNA was used for reverse transcription (RT) with 10 μl of RT reaction mixture containing the thermostable recombinant *Thermus thermophilus* (rTth) enzyme (PE Applied Biosystems). Synthesis of cDNA was carried out with strand-specific primers, sense primer was used to obtain negative-strand RNA, and antisense primer was used to obtain positive-strand RNA, respectively. Reverse transcription was carried out at 70 °C for 15 min. Subsequently, the same primers were used in reverse order in the PCR rounds. Thirty-five cycles of PCR (94 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s) followed by 7 min of extension at 72 °C were carried out on GeneAmp PCR System (PE Applied Biosystems). One-fifth of the PCR products was subjected to electrophoresis on 2% agarose gel and stained with ethidium bromide for observation under UV light. The expected molecular size of PCR products derived from target HCV RNA was 306 bp.

Statistical analysis

The paired *t* test was used to test the significance of the pseudotype VSV entry to MDC. Statistical analyses were performed with the Statview version 4.5 software (Abacus Concepts, Berkeley, CA). A *P* value of less than 0.05 was considered statistically significant.

Acknowledgment

We are grateful to Dr. Keiji Ueda (Osaka University) for providing cord blood cells.

References

Alter, H.J., Purcell, R.H., Shih, J.W., Melpolder, J.C., Houghton, M., Choo, Q.L., Kuo, G., 1989. Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. N. Engl. J. Med. 321 (22), 1494–1500.

Auffermann-Gretzinger, S., Keeffe, E.B., Levy, S., 2001. Impaired dendritic cell maturation in patients with chronic, but not resolved, hepatitis C virus infection. Blood 97 (10), 3171–3176.

Bain, C., Fatmi, A., Zoulim, F., Zarski, J.P., Trepo, C., Inchauspe, G., 2001. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. Gastroenterology 120 (2), 512–524.

Banchereau, J., Steinman, R.M., 1998. Dendritic cells and the control of immunity. Nature 392 (6673), 245–252.

Corado, J., Toro, F., Rivera, H., Bianco, N.E., Deibis, L., De Sanctis, J.B., 1997. Impairment of natural killer (NK) cytotoxic activity in hepatitis C virus (HCV) infection. Clin. Exp. Immunol. 109 (3), 451–457.

Dzionek, A., Fuchs, A., Schmidt, P., Cremer, S., Zysk, M., Miltenyi, S., Buck, D.W., Schmitz, J., 2000. BDCA-2, BDCA-3, and BDCA-4: three markers for distinct subsets of dendritic cells in human peripheral blood. J. Immunol. 165 (11), 6037–6046.

Farci, P., Alter, H.J., Govindarajan, S., Wong, D.C., Engle, R., Lesniewski, R.R., Mushahwar, I.K., Desai, S.M., Miller, R.H., Ogata, N., et al., 1992. Lack of protective immunity against reinfection with hepatitis C virus. Science 258 (5079), 135–140.

Figdor, C.G., van Kooyk, Y., Adema, G.J., 2002. C-type lectin receptors on dendritic cells and Langerhans cells. Nat. Rev., Immunol. 2 (2), 77–84.

Flint, M., Thomas, J.M., Maidens, C.M., Shotton, C., Levy, S., Barclay, W.S., McKeating, J.A., 1999. Functional analysis of cell surface-expressed hepatitis C virus E2 glycoprotein. J. Virol. 73 (8), 6782–6790.

Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., KewalRamani, V.N., Littman, D.R., Figdor, C.G., van Kooyk, Y., 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100 (5), 587–597.

Gerlach, J.T., Diepolder, H.M., Jung, M.C., Gruener, N.H., Schraut, W.W., Zachoval, R., Hoffmann, R., Schirren, C.A., Santantonio, T., Pape, G.R., 1999. Recurrence of hepatitis C virus after loss of virus-specific CD4(+) T-cell response in acute hepatitis C. Gastroenterology 117 (4), 933–941.

Grosjean, I., Caux, C., Bella, C., Berger, I., Wild, F., Banchereau, J., Kaiserlian, D., 1997. Measles virus infects human dendritic cells and blocks their allostimulatory properties for CD4+ T cells. J. Exp. Med. 186 (6), 801–812.

Hart, D.N., 1997. Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90 (9), 3245–3287.

Heckelsmiller, K., Beck, S., Rall, K., Sipos, B., Schlamp, A., Tuma, E., Rothenfusser, S., Endres, S., Hartmann, G., 2002. Combined dendritic cell- and CpG oligonucleotide-based immune therapy cures large murine tumors that resist chemotherapy. Eur. J. Immunol. 32 (11), 3235–3245.

Kaku, H., Goldstein, I.J., Oscarson, S., 1991. Interactions of five D-mannose-specific lectins with a series of synthetic branched trisaccharides. Carbohydr. Res. 213, 109–116.

Kanto, T., Hayashi, N., Takehara, T., Tatsumi, T., Kuzushita, N., Ito, A., Sasaki, Y., Kasahara, A., Hori, M., 1999. Impaired allostimulatory capacity of peripheral blood dendritic cells recovered from hepatitis C virus-infected individuals. J. Immunol. 162 (9), 5584–5591.

Krug, A., Rothenfusser, S., Hornung, V., Jahrsdorfer, B., Blackwell, S., Ballas, Z.K., Endres, S., Krieg, A.M., Hartmann, G., 2001. Identification of CpG oligonucleotide sequences with high induction of IFN-alpha/beta in plasmacytoid dendritic cells. Eur. J. Immunol. 31 (7), 2154–2163.

Lerat, H., Berby, F., Trabaud, M.A., Vidalin, O., Major, M., Trepo, C., Inchauspe, G., 1996. Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J. Clin. Invest. 97 (3), 845–851.

Lerat, H., Rumin, S., Habersetzer, F., Berby, F., Trabaud, M.A., Trepo, C., Inchauspe, G., 1998. In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. Blood 91 (10), 3841–3849.

Liu, Y.J., 2001. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 106 (3), 259–262.

Lozach, P.Y., Lortat-Jacob, H., de Lacroix de Lavalette, A., Staropoli, I., Foung, S., Amara, A., Houles, C., Fieschi, F., Schwartz, O., Virelizier, J.L., Arenzana-Seisdedos, F., Altmeyer, R., 2003. DC-SIGN and L-SIGN are high affinity binding receptors for hepatitis C virus glycoprotein E2. J. Biol. Chem. 278 (22), 20358–20366.

Matsuura, Y., Tani, H., Suzuki, K., Kimura-Someya, T., Suzuki, R., Aizaki, H., Ishii, K., Moriishi, K., Robison, C.S., Whitt, M.A., Miyamura, T., 2001. Characterization of pseudotype VSV possessing HCV envelope proteins. Virology 286 (2), 263–275.

Miller, R.H., Purcell, R.H., 1990. Hepatitis C virus shares amino acid sequence similarity with pestiviruses and flaviviruses as well as members of two plant virus supergroups. Proc. Natl. Acad. Sci. U.S.A. 87 (6), 2057–2061.

Navas, M.C., Fuchs, A., Schvoerer, E., Bohbot, A., Aubertin, A.M., Stoll-Keller, F., 2002. Dendritic cell susceptibility to hepatitis C virus genotype 1 infection. J. Med. Virol. 67 (2), 152–161.

Patterson, S., Rae, A., Hockey, N., Gilmour, J., Gotch, F., 2001. Plasmacytoid dendritic cells are highly susceptible to human immunodeficiency virus type 1 infection and release infectious virus. J. Virol. 75 (14), 6710–6713.

Pileri, P., Uematsu, Y., Campagnoli, S., Galli, G., Falugi, F., Petracca, R.,

Weiner, A.J., Houghton, M., Rosa, D., Grandi, G., Abrignani, S., 1998. Binding of hepatitis C virus to CD81. Science 282 (5390), 938–941.

Pohlmann, S., Zhang, J., Baribaud, F., Chen, Z., Leslie, G.J., Lin, G., Granelli-Piperno, A., Doms, R.W., Rice, C.M., McKeating, J.A., 2003. Hepatitis C virus glycoproteins interact with DC-SIGN and DC-SIGNR. J. Virol. 77 (7), 4070–4080.

Sarobe, P., Lasarte, J.J., Casares, N., Lopez-Diaz de Cerio, A., Baixeras, E., Labarga, P., Garcia, N., Borras-Cuesta, F., Prieto, J., 2002. Abnormal priming of CD4(+) T cells by dendritic cells expressing hepatitis C virus core and E1 proteins. J. Virol. 76 (10), 5062–5070.

Schnorr, J.J., Xanthakos, S., Keikavoussi, P., Kampgen, E., ter Meulen, V., Schneider-Schaulies, S., 1997. Induction of maturation of human blood dendritic cell precursors by measles virus is associated with immunosuppression. Proc. Natl. Acad. Sci. U.S.A. 94 (10), 5326–5331.

Tailleux, L., Schwartz, O., Herrmann, J.L., Pivert, E., Jackson, M., Amara, A., Legres, L., Dreher, D., Nicod, L.P., Gluckman, J.C., Lagrange, P.H., Gicquel, B., Neyrolles, O., 2003. DC-SIGN is the major *Mycobacterium tuberculosis* receptor on human dendritic cells. J. Exp. Med. 197 (1), 121–127.

Wedemeyer, H., He, X.S., Nascimbeni, M., Davis, A.R., Greenberg, H.B., Hoofnagle, J.H., Liang, T.J., Alter, H., Rehermann, B., 2002. Impaired effector function of hepatitis C virus-specific CD8+ T cells in chronic hepatitis C virus infection. J. Immunol. 169 (6), 3447–3458.

Weiner, A., Erickson, A.L., Kansopon, J., Crawford, K., Muchmore, E., Hughes, A.L., Houghton, M., Walker, C.M., 1995. Persistent hepatitis C virus infection in a chimpanzee is associated with emergence of a cytotoxic T lymphocyte escape variant. Proc. Natl. Acad. Sci. U.S.A. 92 (7), 2755–2759.

Wu, L., Martin, T.D., Vazeux, R., Unutmaz, D., KewalRamani, V.N., 2002. Functional evaluation of DC-SIGN monoclonal antibodies reveals DC-SIGN interactions with ICAM-3 do not promote human immunodeficiency virus type 1 transmission. J. Virol. 76 (12), 5905–5914.